Transcription factor NRF2 protects mice against dietary iron-induced liver injury by preventing hepatocytic cell death

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Originally published in Journal of Hepatology 60(2), 354-61, September 7, 2014.

Doi: 10.1016/j.jhep.2013.09.004

KEYWORDS: Hepatocyte; Hemochromatosis; Antioxidant; Mito-TEMPOL; 8-Hydroxy-20-deoxyguanosine; Necrosis.

Background & Aims: The liver, being the major site of iron storage, is particularly exposed to the toxic effects of iron. Tran- scription factor NRF2 is critical for protecting the liver against disease by activating the transcription of genes encoding detoxi-fication/antioxidant enzymes. We aimed to determine if the NRF2 pathway plays a significant role in the protection against hepatic iron overload.
Methods: Wild type and Nrf2$^{-/-}$ mouse primary hepatocytes
Wereincubated with ferric ammonium citrate. Wild type and Nrf2$^{-/-}$ mice were fed standard rodent chow or iron-rich diet for 2 weeks, with or without daily injection of the antioxidant mito-TEMPOL.
Results: In mouse hepatocytes, iron induced the nuclear translo- cation of NRF2 and the expression of cytoprotective genes in an NRF2-dependent manner. Moreover, Nrf2$^{-/-}$ hepatocytes were highly susceptible to iron-induced cell death. Wild-type and Nrf2$^{-/-}$ mice fed iron-rich diet accumulated similar amounts of iron in the liver and were equally able to increase the expression of hepatic hepcidin and ferritin. Nevertheless, in Nrf2-null mice the iron loading resulted in progressive liver injury, ranging from mild confluent necrosis to severe necroinflammatory lesions. Hepatocytic cell death was associated with gross ultrastructural damage to the mitochondria. Notably, liver injury was prevented in iron-fed animals that received mito-TEMPOL.
Conclusions: NRF2 protects the mouse liver against the toxicity of dietary iron overload by preventing hepatocytic cell death. We identify NRF2 as a potential modifier of liver disease in iron overload pathology and show the beneficial effect of the antiox- idant mito-TEMPOL in a mouse model of dietary iron-induced liver injury.

INTRODUCTION

Iron is a component of several metalloproteins involved in crucial metabolic processes such as oxygen sensing and transport, energy metabolism and DNA synthesis. However, iron in excess is detrimental, as it can catalyze the formation of damaging radical species via Fenton-type reactions [1]. In normal conditions, iron toxicity is prevented by the binding of extracellular iron in the plasma to transferrin and by storage of intracellular iron in a protein with high storage capacity, ferritin [2]. In addition, plasma iron levels are tightly regulated by the action of the peptide hormone hepcidin. Hepcidin, which is mostly secreted in the liver, promotes the degradation of the iron exporter ferroportin expressed on the surface of iron-releasing cells, thus reducing intestinal iron absorption and its mobilization from hepatic stores, and promoting iron retention within erythrophagocyto- sing macrophages [2]. Nevertheless, mutations in the genes encoding hepcidin (Hamp) or other key regulatory proteins like HFE, hemojuvelin or transferrin receptor 2 result in inappropriately low hepcidin levels and lead to the development of Hereditary Hemochromatosis (HH), an autosomal recessive disorder that is characterized by excessive absorption of dietary iron and its deposition in the parenchymal cells of the liver and other body organs [3]. The liver, in particular, has the capacity to accumulate a great amount of iron and is thus particularly exposed to its toxic effects [4]. Cellular injury is presumably caused by oxygen radical-mediated damage to cellular organelles, leading to hepatocyte death. The HH disease penetrance is variable though. Whilst the chronic accumulation of iron in the organism per se is reasonably well tolerated in most individuals, some HH patients develop liver fibrosis and cirrhosis, and in some cases primary hepatocellular carcinoma [3]. In rodent models, long-term iron supplementation per se fails to induce significant liver damage [5, 6], suggesting that under normal conditions organisms activate mechanisms that allow an adaptation to increased oxidative insult.
Transcription factor Nuclear factor-erythroid 2-related factor 2 (Nfe2l2/Nrf2) is a bZIP redox-sensitive transcription factor that regulates the transcriptional induction of a battery of antioxidant response element (ARE)-containing genes in response to cellular stresses. There is growing evidence that NRF2 is an important modifier of diseases involving oxidative stress (e.g. inflammatory and neurodegenerative diseases, and cancer) [7] and the liver of $Nrf2^{-/-}$ mice is reportedly more susceptible to oxidative and electrophilic stress [8]. However, the protective role of NRF2 in iron overload disease remains undetermined. Here, we investigated whether NRF2 is critical for the protection of liver parenchymal cells against iron toxicity in vitro and in vivo.

MATERIALS AND METHODS

Animals and experimental design

C57BL/6J mice and $Nrf2^{-/-}$ mice on a C57BL/6J background [9] (Riken, Japan) were housed and bred at the 'Instituto de Biologia Molecular e Celular' animal facility. Male mice (16 week-old) were fed standard rodent chow (Global rodent diet, Harlan Laboratories, Barcelona, Spain) or iron-rich diet (Global rodent diet supplemented with 2.0% carbonyl iron) for 2 weeks. In a subsequent experiment, $Nrf2^{-/-}$ animals were maintained on the iron-rich diet for the same time period and injected daily with either (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylami- no)-2-xoethyl) triphenylphosphonium chloride monohydrate (mito-TEMPOL, Santa Cruz Biotechnology, Heidelberg, Germany) (10 mg/kg intraperitoneally) or saline (control), starting from the day before the start of dietary supplementation. At the end of the dietary treatment, mice were anaesthetized, blood was collected by retro-orbital bleeding, and the animals were sacrificed for organ collection.
Since the saline injections in $Nrf2^{-/-}$ animals on iron-rich diet had no effect on any of the parameters assessed when comparing with $Nrf2^{-/-}$ animals on the same diet, samples from both groups were pooled for statistical analysis.

Cell cultures

Primary hepatocyte cultures from C57BL/6 ($Nrf2^{+/+}$) and $Nrf2^{-/-}$ mice were prepared as described in Supplementary data.
For a full description of Materials and methods, see Supplementary data.

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RESULTS

NRF2 activation protects mouse hepatocytes from iron toxicity

Under oxidative stress, NRF2 escapes proteasomal degradation and is translocated to the nucleus, where it activates the tran- scription of a battery of ARE-containing cytoprotective genes [7]. The activation of NRF2 by iron was investigated in primary hepatocyte cultures derived from wild-type and Nrf2$^{-/-}$ mice that were incubated with ferric ammonium citrate (FAC), a source of inorganic iron that is rapidly taken up by cultured cells and mimics the non-transferrin-bound iron found in the plasma of hemochromatosis patients [10,11]. By immunofluorescence, we show an increase of NRF2 in the nuclei of wild-type hepatocytes at the end of 17 h of incubation with FAC (1.71 μg/ml) (Fig. 1A).

We also monitored the mRNA expression of three prototypical NRF2 target genes in hepatocytes exposed to FAC. In basal conditions, wild-type hepatocytes expressed higher levels of Nqo1, Gclc, and Gsta1 than Nrf2 $^{-/-}$ cells. Incubation with FAC for 24 h significantly increased the steady-state levels of these genes in wild-type hepatocytes but not in Nrf2$^{-/-}$ cells, showing that NRF2 is required for their transcriptional activation by iron (Fig. 1B).

After demonstrating that NRF2 is activated by iron in wild- type hepatocytes, we assessed whether Nrf2$^{-/-}$ hepatocytes were more susceptible to iron toxicity. Cells were incubated with increasing concentrations of FAC or ammonium citrate (AC) as control for 24 h and their viability was assessed by the MTT assay, which measures the formation of a water-insoluble MTT formazan by mitochondrial dehydrogenases of living cells. As depicted in Fig. 1C, AC had no effect on the activity of hepatocytic mitochondrial dehydrogenases in either wild type or Nrf2$^{-/-}$ cells. While wild-type hepatocytes were resistant to all concentrations of FAC, a dramatic, dose-dependent reduction in the activity of mitochondrial dehydrogenases was observed in Nrf2$^{-/-}$ hepatocytes. In the latter, FAC treatment caused morphological alterations that included rounding up of hepatocytes (Supplementary Fig. 1). We then performed a propidium iodide (PI) uptake assay that measures the loss of cytoplasmic membrane integrity in dead cells. Incubation with FAC (1.714 μg/ml) caused a significant increase in the number of PI-positive nuclei in Nrf2$^{-/-}$ hepatocytes but not their wild-type counterparts (Fig. 1D). We inspected the nuclear morphology of PI-positive cells for the presence of apoptotic bodies. As a positive control, we incubated Nrf2$^{-/-}$ cells with a concentration of staurosporine that induces apoptosis in mouse primary hepatocytes [12]. Staurosporine- treated cells displayed chromatin margination and condensation, two typical features of apoptotic cells. In Nrf2$^{-/-}$ hepatocytes incubated with FAC, however, we did not observe any nuclei with the typical features of apoptosis (Fig. 1E), which suggests that iron kills Nrf2$^{-/-}$ hepatocytes by primary necrosis rather than apoptosis.

Finally, we investigated the formation of 8-hydroxy-2'-deoxy- guanosine (8-OHdG), a product of oxidatively damaged DNA formed by hydroxyl radical or singlet oxygen [13], in mouse hepatocytes incubated with iron. DNA damage was measured using the alkaline comet assay as human 8-hydroxyguanine DNA glycosylase 1 (hOGG1)-sensitive sites. hOGG1 is considered to detect 8-OHdG in cell culture experiments with great specificity [14]. Fig. 1F shows the level of hOGG1 sensitive sites in hepatocyte cultures after 24 h exposure to FAC. Notably, iron increased hOGG1-sensitive sites exclusively in Nrf2$^{-/-}$ hepatocytes, which suggests that the latter are more susceptible to iron-induced oxidative stress.

NRF2 protects mice against dietary iron-induced liver injury

C57BL/6 (wild-type) and Nrf2$^{-/-}$ mice were either fed standard rodent chow or a diet containing 2.0% carbonyl iron (iron-rich diet) ad libitum for 2 weeks. No significant differences in the relative liver weight were found. The levels of non-heme liver iron in animals of both strains under standard diet were not significantly different either. Feeding carbonyl iron increased the hepatic iron stores, transferrin saturation and serum iron levels significantly, irrespective of the Nrf2 status (Table 1). Significant increases in hepatic total ferritin levels (Fig. 2A) and in the steady-state mRNA levels of Hamp and Hamp2 were also observed in both wild-type and Nrf2$^{-/-}$ mice fed iron-rich diet (Fig. 2B). We conclude that the lack of Nrf2 had no impact on the hepatic iron loading or on the ability of the liver to respond by activating key regulators of iron homeostasis.

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We then determined whether the hepatic iron loading altered the expression of Nrf2 or of its target genes. As expected, wild- type mice expressed significantly higher levels of Nrf2, Nqo1, and Gsta1 mRNA at steady-state in the liver (Fig. 2C). Whilst the steady-state mRNA levels of Nrf2 were not altered by dietary iron, the iron-rich diet caused a significant increase in hepatic Nqo1 and Gsta1 mRNA levels in wild-type but not in Nrf2$^{-/-}$ mice, demonstrating that these genes are regulated by dietary iron in an NRF2-dependent manner (Fig. 2C). NQO1 protein levels also increased in the livers of wild-type but not Nrf2$^{-/-}$ mice under iron-rich diet (Fig. 2D).

The next step was to determine if the lack of NRF2 would affect the capacity of mice to handle the iron overload in comparison with wild-type animals. Whilst we found no major alterations in the spleen, heart, pancreas or duodenum (data not shown), the analysis of liver histology revealed striking differences between wild-type and Nrf2$^{-/-}$ mice in what concerns the preservation of liver architecture and the pattern of iron deposition (Fig. 3A). As expected, none of the animals on normal diet displayed positive Perls' Prussian blue staining for hepatic iron. In wild-type mice fed iron-rich diet, iron deposition occurred predominantly in the hepatocytes adjacent to the portal tracts and in some animals also in midzone hepatocytes (sidero- sis grades 1–2), sparing the Kupffer cells, which mimics the typical pattern of iron deposition found in HH patients. In Nrf2$^{-/-}$ mice, hepatocellular siderosis extended from the periportal regions to reach midzone or centrilobular regions (siderosis grades 1–3), and was often less granular (Fig. 3A).

Liver sections stained with haematoxylin and eosin (H&E) revealed the existence of confluent areas of necrosis in most Nrf2$^{-/-}$ mice fed iron-rich diet, ranging from focal confluent necrosis (necrosis grade 1) to panacinar or multiacinar necrosis (grade 6) (Fig. 3A). Hepatocytic necrosis was evidenced by the acidophilic degeneration of hepatocytes with nuclei in advanced stage of karyolysis or karyorrhexis, and by the presence of hepatocytes with swollen cytoplasm (ballooning degeneration of the hepatocytes) (Fig. 3A and Supplementary Fig. 2). Apoptotic cells were occasionally found in periportal areas, but their number was greatly outnumbered by those presenting the typical features of oncotic necrosis. Hemorrhage was observed in the individuals with the highest necrosis scores (Supplementary Fig. 2).

Necrotic and apoptotic cell death result in distinct ultrastructural changes to hepatocellular membranes and organelles. Electron microscopy showed that periportal hepatocytes of wild-type and Nrf2$^{-/-}$ mice fed normal diet presented abundant glycogen and rough endoplasmic reticulum (RER), and regularly shaped mitochondria with intact cristae (Fig. 3B). In hepatocytes from wild-type mice fed iron-rich diet we found an abundance of secondary lysosomes, most of which containing electron-dense (presumably iron-rich) material. No major changes were observed regarding the morphology of mitochondria or the RER (Fig. 3B). Hepatocytes of Nrf2$^{-/-}$ animals fed iron-rich diet also displayed evidence of lysosomal activation but the most prominent feature was the diffuse cellular edema (oncosis) affecting the totality of hepatocytes. There was gross damage to the mitochondria, which appeared swollen, with the cristae in various stages of disintegration. In some hepatocytes, we observed swelling of the inner membrane-matrix compartment, whereas in others there was rupture of the outer mitochondrial membrane. Proliferation of smooth endoplasmic reticulum (SER) and dilated RER with loss of ribosomes were observed (Fig. 3B and C). We found evidence of autolytic cell destruction in disintegrating (necrotic) hepatocytes, including the loss of cytoplasmic and organelle membrane integrity, and the presence of amorphous cytoplasm (Fig. 3C). Hepatocytic necrosis often results in acute inflammatory reaction [15] and accordingly hepatocytic cell death was associated with dense inflammatory infiltrates streaming out from the portal tri- ads (Fig. 3A and Supplementary Fig. 2), which included macro- phages, lymphocytes and polymorphonuclear cells (Fig. 3C, left panel). Lobular inflammation was confirmed by a significant increase in the number of CD45^{+} leukocytes (Fig. 4A). The association of these acute inflammatory infiltrates with hepatocytic necrosis was illustrated by the extensive TUNEL staining of contiguous hepatocytes, indicative of DNA fragmentation (Fig. 4B). We observed both nuclear and cytoplasmic staining of swollen cells, as typically seen in cells undergoing oncotic necrosis due to the release of nucleases from infiltrating inflammatory cells [16–18]. We also evaluated the levels of cleaved caspase 3, a critical executioner of apoptosis [19]. As depicted in Supplementary further supports the predominance of necrotic rather than apoptotic cell death. Accordingly, we found a significant rise in the activity of the serum transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are released by injured hepatocytes, in Nrf2$^{-/-}$ mice fed iron-rich diet (Fig. 4D). Finally, we found a significant elevation in the number of 8-OHdG immunoreactive nuclei in perivascular

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hepatocytes from Nrf2$^{-/-}$ mice fed iron-rich diet (Fig. 4C), indicating that the livers of these animals were under oxidative stress.
The mitochondria are the major site of intracellular reactive oxygen species (ROS) production and excessive iron may lead to mitochondrial dysfunction [20], which could result in the leakage of mitochondrial ROS into the cytosol. The mitochondria of hepatocytes from Nrf2$^{-/-}$ mice fed iron-rich diet were swollen (Fig. 3B and C), which is known to cause the onset of mitochondrial permeability transition (MPT). MPT, in turn, is a cause of both apoptotic and necrotic hepatocytic cell death, via the release of pro-apoptotic factors and a reduction in cellular ATP, respectively [17,21]. To determine if the mitochondria played a significant role in the increased susceptibility of Nrf2$^{-/-}$ hepatocytes to dietary iron overload, a group of Nrf2$^{-/-}$ mice on iron-rich diet received daily injections of mito-TEMPOL (10 mg/kg), a derivative of the antioxidant TEMPOL that is targeted to the mitochondria [22,23]. The antioxidant therapy did not affect the uptake of iron from the diet or its deposition in the liver, as the animals displayed similar grading of hepatic siderosis (Fig. 3A) and similar levels of hepatic non-heme iron, serum iron and transferrin saturation to those of Nrf2$^{-/-}$ mice on the same diet that did not receive mito-TEMPOL (Table 1). However, the necrosis scores were similar to those of Nrf2$^{-/-}$ mice on normal diet (Fig. 3A). Histological (Fig. 3A) and ultrastructural (Fig. 3B) analysis showed that the antioxidant therapy prevented hepatocytic necrosis. We found no evidence of lobular inflammation (Fig. 4A), hepatocytic DNA fragmentation (Fig. 4B) or oxidation (Fig. 4C) in these animals, and the levels of serum ALT and AST were not significantly different from those of animals on normal diet (Fig. 4D). In summary, we demonstrate that the hepatotoxicity of dietary iron towards Nrf2$^{-/-}$ animals is abrogated via the use of an antioxidant targeted to the mitochondria.

DISCUSSION

In HH patients, a genetic predisposition towards excessive absorption of dietary iron causes iron accumulation in tissues. In some individuals, hepatic iron overload results in liver injury, presumably as a result of the contribution of environmental or genetic risk factors. Alcohol consumption is a well-recognized environmental risk factor [3], but genetic factors remain poorly understood. So far the search for genetic modifiers has focused on the identification of modifiers of iron loading rather than of genetic determinants of protection against excessive iron.
Transcription factor NRF2 coordinates the adaptation of cells and organisms to oxidative stress [7]. Previous work showed that Nrf2$^{-/-}$ mice are more susceptible to ferric nitrilotriacetate- induced renal toxicity [24]. Here, we focused on the hepatotoxiccytochrome P450–dependent metabolism [26]. We have also investigated the ability of hepatocytes and liver from Nrf2-null mice to tolerate iron excess. We show for the first time that the activation of NRF2 signaling by iron is associated with protection against hepatocytic cell death in vitro and in vivo. The fact that Nrf2$^{-/-}$ mice fed iron-rich diet develop marked liver injury despite a similar hepatic iron loading to wild-type animals is of great relevance because although the liver is particularly exposed to the toxic effects of iron in excess, rodents are generally resistant to iron-induced liver injury. Mouse models of HH develop the spontaneous iron overload phenotype seen in human patients without developing significant iron-induced liver injury [27,28]. Likewise, the supplementation of mouse diets with carbonyl iron is generally well tolerated, despite the substantial increase in hepatic iron [5,29]. In our view, this is because wild-type hepatocytes are equipped with efficient antioxidant defenses that make them resistant to iron-induced ROS. Swelling of hepatocytes, causing ALT/AST release, as observed in Nrf2$^{-/-}$ mice fed iron-rich diet, are typical features of ROS-induced necrosis in vivo [18]. Further evidence of iron-mediated oxidative stress is provided by the increased levels of a specific marker of oxidative DNA damage (8- OHdG) in hepatocytes from Nrf2$^{-/-}$ mice fed iron-rich diet and the abrogation of the hepatotoxicity of dietary iron via the use of an antioxidant that is targeted to the mitochondria (mito-TEMPOL), which is capable of trapping free radicals and inhibiting the catalytic action of Fe^{2+} [30].
A striking feature of necrotic hepatocytes from Nrf2$^{-/-}$ mice fed iron-rich diet was the extreme swelling of mitochondria. In hepatocytes, non-heme iron that is initially sequestered in lysosomes may be released and taken up into mitochondria, where it catalyzes toxic ROS-mediated reactions [31]. This may open nonspecific permeability transition pores in the mitochondrial inner membrane that cause mitochondrial membrane depolarization, uncoupling of oxidative phosphorylation, and large scale mitochondrial swelling, leading to hepatocyte necrosis from

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adenosine triphosphate depletion. Notably, an extreme swelling of mitochondria is also found in severe HH patients [32].
Hepatocytic cell death in Nrf2$^{-/-}$ mice fed iron-rich diet was also favored by the development of severe portal inflammation. Oncotic necrosis of hepatocytes causes cytoplasmic swelling and progressive disintegration of cytoplasmic membranes. The release of material from necrotizing cells into the extracellular compartment often results in an inflammatory reaction [15].
In conclusion, we describe the Nrf2$^{-/-}$ mouse fed iron-rich diet as a valid model for the study of dietary iron-mediated liver injury and identify NRF2 as a potential modifier of liver disease in iron overload pathology. We anticipate that resistance to oxidative stress may be a modifier of disease progression in HH and other iron overload diseases. Functional polymorphisms in Nrf2 have been described, which may lead to loss of function. Based on our current results, it is tempting to speculate that individuals with a genetic predisposition to develop iron overload (such as HH patients) who also carry the Nrf2 single nucleotide polymorphisms would be at higher risk of developing iron overload-related pathology. This possibility should warrant great attention in the future.

Financial support

This work is funded by FEDER Funds through the Operational Competitiveness Programme – COMPETE and by National Funds through FCT – Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-011062 (PTDC/SAU-FCF/ 101177/2008). TLD is supported by ''Programa Ciência – financiado pelo POPH – QREN – Tipologia 4.2 – Promoção do Emprego Científico, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES''.

Conflict of interest
The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgements
The authors are grateful to Rui Fernandes for technical assistance in TEM, José Alberto Duarte for the interpretation of TEM images, Nádia Duarte and Vanessa Zuzarte-Luís for advice on the establishment of mouse primary hepatocyte cultures, and Cátia Silva for assistance in liver histopathology analysis.

Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013. 09.004.

References

[1] Papanikolaou G, Pantopoulos K. Iron metabolism and toxicity. Toxicol Appl Pharmacol 2005; 202:199–211.
[2] Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. Cell 2010; 142:24–38.
[3]Pietrangelo A. Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. Gastroenterology 2010; 139:393–408, [408 e391–e392].
[4] Batts KP. Iron overload syndromes and the liver. Mod Pathol 2007; 20:S31–S39.
[5]Pigeon C, Turlin B, Iancu TC, Leroyer P, Le Lan J, Deugnier Y, et al. Carbonyl- iron supplementation induces hepatocyte nuclear changes in BALB/CJ male mice. J Hepatol 1999; 30:926–934.
[6]Khan MF, Wu X, Tipnis UR, Ansari GA, Boor PJ. Protein adducts of malondialdehyde and 4-hydroxynonenal in livers of iron-loaded rats: quantitation and localization. Toxicology 2002; 173:193–201.
[7]Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environ- mental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007; 47:89–116.
[8]Klaassen CD, Reisman SA. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. Toxicol Appl Pharmacol 2010;244:57–65.

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[9] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997; 236:313–322.
[10] Hershko C, Peto TE. Non-transferrin plasma iron. Br J Haematol 1987; 66:149–151.
[11] Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ. Non- transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatog- raphy and nuclear magnetic resonance spectroscopy. J Biol Chem 1989; 264:4417–4422.
[12] Feng G, Kaplowitz N. Mechanism of staurosporine-induced apoptosis in murine hepatocytes. Am J Physiol Gastrointest Liver Physiol2002; 282:G825–G834.
[13] Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Yoshida A, Uchida K, et al.
Quantitative immunohistochemical determination of 8-hydroxy-20 -deoxy- guanosine by a monoclonal antibody N45.1: its application to ferricnitrilotriacetate-induced renal carcinogenesis model. Lab Invest 1997; 76:365–374.
[14] Smith CC, O'Donovan MR, Martin EA. HOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. Mutagenesis 2006; 21:185–190.
[15] Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 2010; 11:700–714.
[16] Gujral JS, Knight TR, Farhood A, Bajt ML, Jaeschke H. Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? Toxicol Sci 2002; 67:322–328.
[17] Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 2006; 43:S31–S44.
[18] Hong JY, Lebofsky M, Farhood A, Jaeschke H. Oxidant stress-induced liver injury in vivo: role of apoptosis, oncotic necrosis, and c-Jun NH2-terminal kinase activation. Am J Physiol Gastrointest Liver Physiol 2009; 296:G572–G581.
[19] Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 2010; 11:621–632.
[20] Gao X, Qian M, Campian JL, Marshall J, Zhou Z, Roberts AM, et al. Mitochondrial dysfunction may explain the cardiomyopathy of chronic iron overload. Free Radic Biol Med 2010; 49:401–407.
[21] Rodriguez-Enriquez S, He L, Lemasters JJ. Role of mitochondrial permeability transition pores in mitochondrial autophagy. Int J Biochem Cell Biol 2004; 36:2463–2472.
[22] Trnka J, Blaikie FH, Logan A, Smith RA, Murphy MP. Antioxidant properties of MitoTEMPOL and its hydroxylamine. Free Radic Res 2009; 43:4–12.
[23] Dikalova AE, Bikineyeva AT, Budzyn K, Nazarewicz RR, McCann L, Lewis W, et al. Therapeutic targeting of mitochondrial superoxide in hypertension. Circ Res 2010; 107:106–116.
[24] Kanki K, Umemura T, Kitamura Y, Ishii Y, Kuroiwa Y, Kodama Y, et al. A possible role of nrf2 in prevention of renal oxidative damage by ferric nitrilotriacetate. Toxicol Pathol 2008; 36:353–361.
[25] Moon MS, McDevitt EI, Zhu J, Stanley B, Krzeminski J, Amin S, et al. Elevated hepatic iron activates NF-E2-related factor 2-regulated pathway in a dietary iron overload mouse model. Toxicol Sci 2012; 129:74–85.
[26] Moon MS, Richie JP, Isom HC. Iron potentiates acetaminophen-induced oxidative stress and mitochondrial dysfunction in cultured mouse hepato- cytes. Toxicol Sci 2010; 118:119–127.
[27] Finberg KE, Whittlesey RL, Andrews NC. Tmprss6 is a genetic modifier of the Hfe-hemochromatosis phenotype in mice. Blood 2011; 117:4590–4599.
[28] Delima RD, Chua AC, Tirnitz-Parker JE, Gan EK, Croft KD, Graham RM, et al. Disruption of hemochromatosis protein and transferrin receptor 2 causes iron-induced liver injury in mice. Hepatology 2012; 56:585–593.
[29] Arezzini B, Lunghi B, Lungarella G, Gardi C. Iron overload enhances the development of experimental liver cirrhosis in mice. Int J Biochem Cell Biol 2003; 35:486–495.
[30] Monti E, Cova D, Guido E, Morelli R, Oliva C. Protective effect of the nitroxide tempol against the cardiotoxicity of adriamycin. Free Radic Biol Med 1996;21:463–470.
[31] Uchiyama A, Kim JS, Kon K, Jaeschke H, Ikejima K, Watanabe S, et al. Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury. Hepatology 2008; 48:1644–1654.
[32] Iancu TC, Deugnier Y, Halliday JW, Powell LW, Brissot P. Ultrastructural sequences during liver iron overload in genetic hemochromatosis. J Hepatol 1997; 27:628–638.

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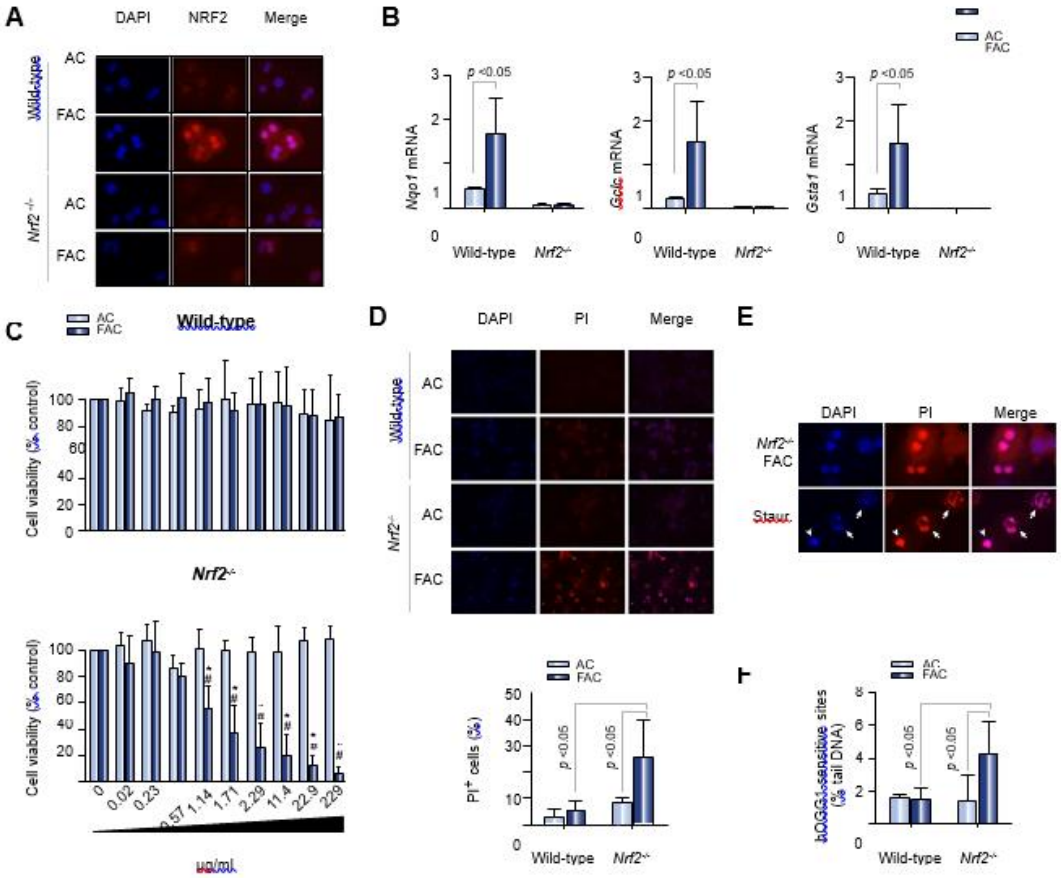

Fig. 1. NRF2 is activated by iron and protects mouse primary hepatocytes from iron toxicity. Unless stated otherwise, hepatocytes were incubated with 1.71 μg/ml FAC or AC for 24 h. (A) Immunofluorescence detection of NRF2 at 17 h of incubation. The increased fluorescence in nuclei of wild-type hepatocytes treated with FAC is representative of 3 independent experiments. (B) The mRNA levels of cytoprotective genes were assessed by real-time RT-PCR. (C) Hepatocyte viability was measured with the MTT assay. *p <0.05 when compared with *Nrf2*$^{-/-}$ AC, #p <0.05 when compared with wild-type FAC. (D) Hepatocytes were stained with the DNA-binding fluorochrome propidium iodide, which is impermeable to the normal plasma membrane but stains nuclei of necrotic and late apoptotic cells. Nuclei were counterstained with DAPI. (E) Fluorescence micrographs showing representative nuclei of *Nrf2*$^{-/-}$ hepatocytes incubated with FAC or staurosporine [Staur.] (5 μM). Note the typical features of apoptosis in staurosporine-treated cells, including chromatin margination (arrows) and condensation (arrowheads). (F) Oxidatively modified DNA was measured using the alkaline comet assay as hOGG1-sensitive sites. (This figure appears in color on the web.)

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Table 1. Iron loading parameters in mice fed normal or iron-rich diet.

	Sample size	Relative liver weight	Liver non-heme iron (µg)	Serum iron (µg/ml)	Transferrin saturation (%)
B6					
Normal	8	0.049 ± 0.003	189.5 ± 28.1	129.6 ± 17.1	39.7 ± 3.8
Iron-rich	6	0.047 ± 0.003	621.6 ± 102.7[a]	235.5 ± 23.6[a]	91.4 ± 0.7[a]
Nrf2$^{-/-}$					
Normal	8	0.042 ± 0.005	145.3 ± 30.4	141.8 ± 10.4	39.1 ± 1.5
Iron-rich	12	0.045 ± 0.011	632.6 ± 141.1[a]	230.9 ± 28.7[a]	93.1 ± 4.7[a]
Iron-rich + mito-TEMPOL	6	0.036 ± 0.003	584.9 ± 186.0[a,b]	252.0 ± 21.4[a,b]	94.9 ± 2.1[a,b]

[a] p <0.001 *vs*. animals of the same genotype on normal diet.
[b] p >0.05 *vs*. *Nrf2*$^{-/-}$ animals on iron-rich diet.

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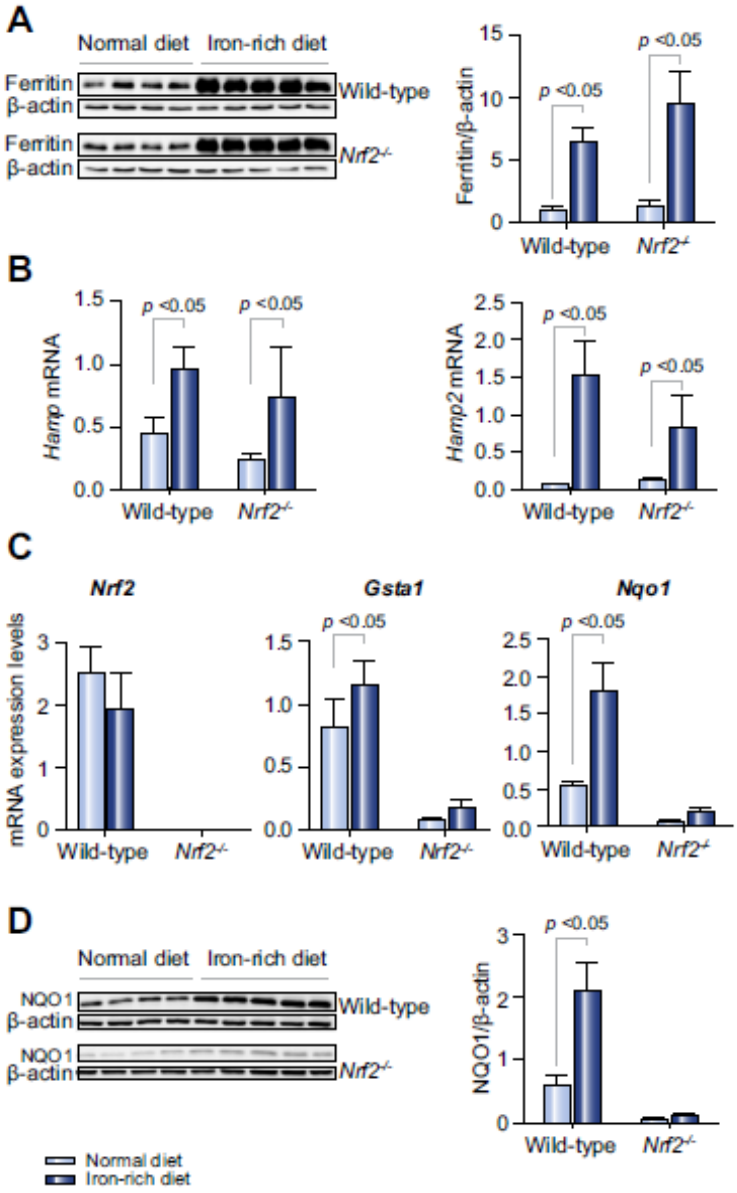

Fig. 2. Activation of key iron homeostasis genes and antioxidant/cytoprotective genes in the liver of mice fed iron-rich diet. Wild-type and *Nrf2*$^{-/-}$ mice were fed standard rodent chow or iron-rich diet for 2 weeks. (A) Western blot and densitometric quantification of total ferritin in liver extracts. (B) Transcriptional levels of the two genes encoding mouse hepcidin were determined by real-time RT-PCR. (C) The mRNA expression levels of *Nrf2, Gsta1,* and *Nqo1* were assessed by real-time RT-PCR. (D) Western blot and densitometric quantification of NQO1 in liver extracts.

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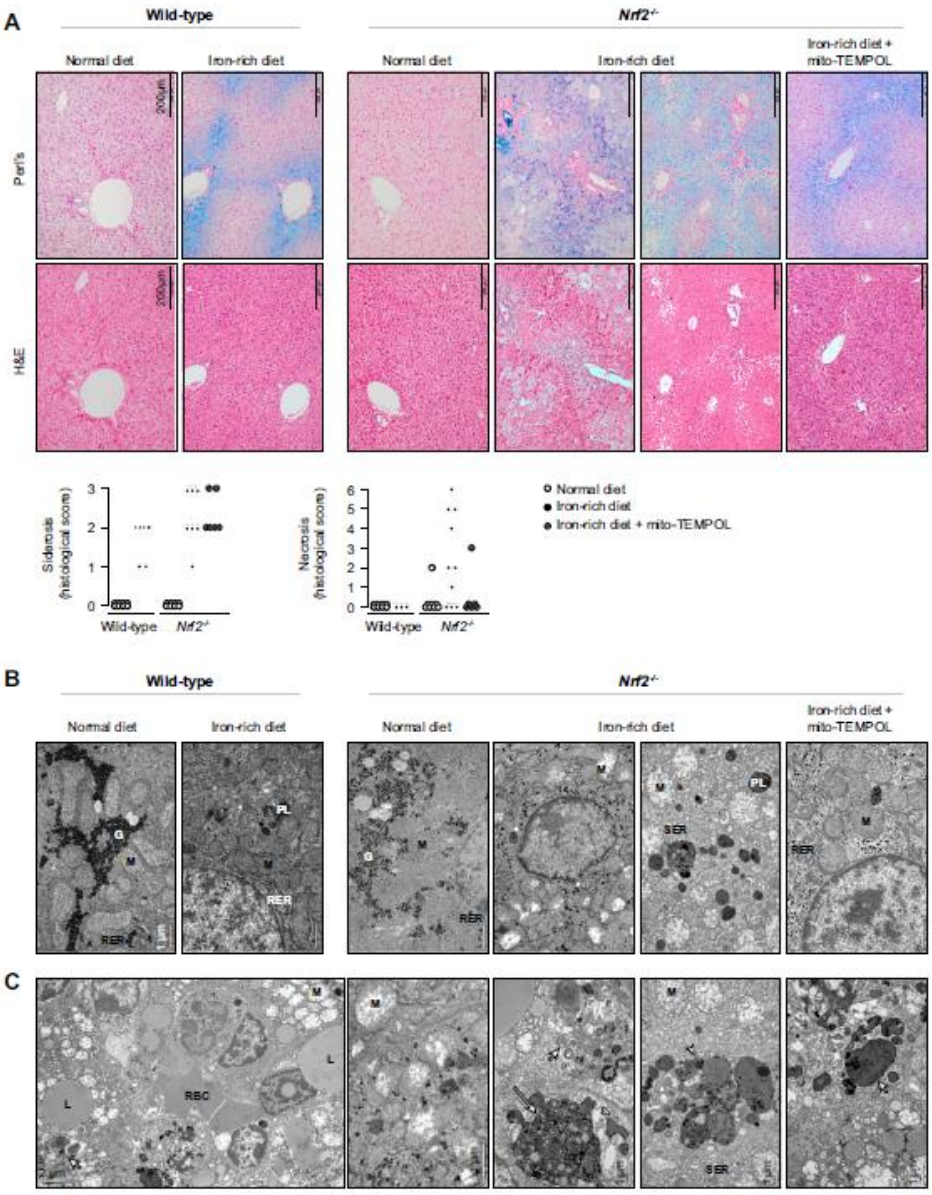

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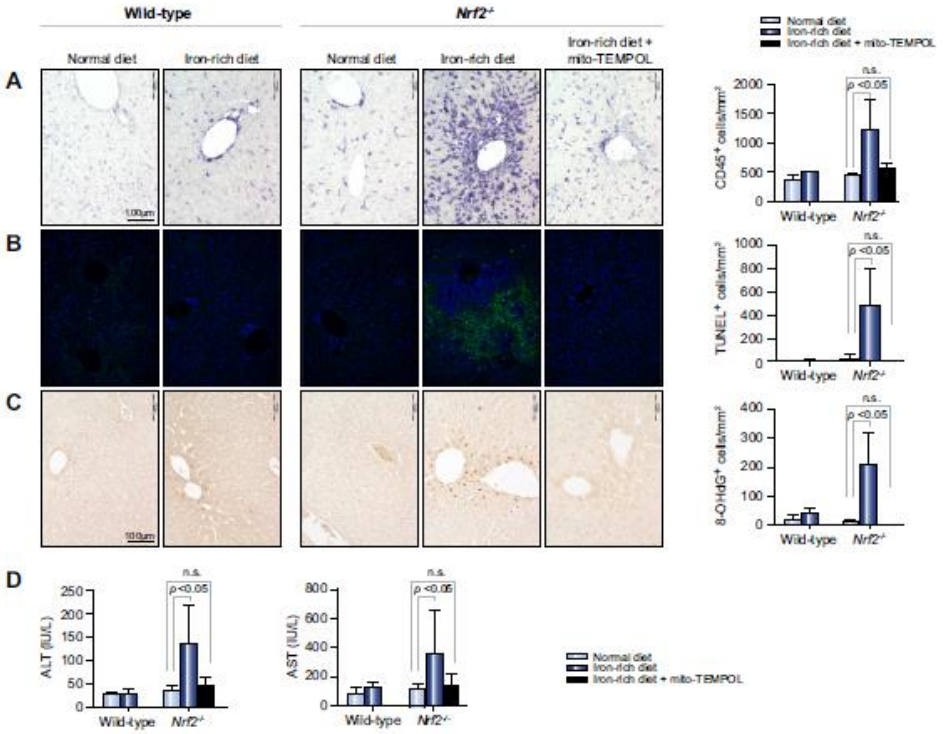

Fig. 4. Evidence of lobular inflammation, hepatotocytic cell death and oxidative DNA damage in *Nrf2*$^{-/-}$ mice fed iron-rich diet. Wild-type and *Nrf2*$^{-/-}$ mice were fed standard rodent chow or iron-rich diet for 2 weeks. A group of *Nrf2*$^{-/-}$ animals on iron-rich diet received daily injections of the antioxidant mito-TEMPOL. Representative images and quantification of CD45+ leukocytes (A), TUNEL-positive hepatocytes (B), and hepatocytic nuclei immunoreactive for 8-hydroxy-20-deoxyguanosine (8-OHdG) (C) in liver tissues are depicted. (D) ALT and AST activities were measured in mouse serum. n.s., not significant. (This figure appears in color on the web.)

Fig. 3. Histopathological and ultrastructural features in the liver of *Nrf2*$^{-/-}$ animals fed iron-rich diet. Wild-type and *Nrf2*$^{-/-}$ mice were fed standard rodent chow or iron-rich diet for 2 weeks. A group of *Nrf2*$^{-/-}$ animals on iron-rich diet received daily injections of the antioxidant mito-TEMPOL. (A) Representative liver sections and grading of hepatic siderosis and necrosis. Note the swollen and eosinophilic hepatocytes, and confluent areas of necrosis in *Nrf2*$^{-/-}$ mice fed iron-rich diet, whereas in all other experimental groups the liver was histologically normal with no change in the lobular architecture. (B) TEM analysis of periportal hepatocytes denoting abundant glycogen (G), rough endoplasmic reticulum (RER) and mitochondria (M) in animals fed normal diet and the accumulation of electron-dense material within phagolysosomes (PL) in animals fed iron-rich diet. Hepatocytes from *Nrf2*$^{-/-}$ mice fed iron-rich diet showed gross mitochondrial swelling with decreased matrix density and outer membrane rupture, and hypertrophied smooth endoplasmic reticulum (SER). Note the normal mitochondrial morphology in the *Nrf2*$^{-/-}$ animals fed iron-rich diet that received mito-TEMPOL. (C) Ultrastructural features of hepatocytic cell death in *Nrf2*$^{-/-}$ animals fed iron-rich diet are depicted, including disintegrating (necrotic) hepatocytes containing membranous phagolysosomes and displaying plasma membrane disruption (small arrow), a giant secondary lysosome containing electron-dense material and lipofuscin (long arrow), and an 'acidophilic' body formed from a dying hepatocyte where fat droplets, disintegrated nucleus, mitochondria and phagolysosomes are still recognizable (small arrowhead). Lipid deposition (L) and infiltration of red blood cells (RBC) and inflammatory cells were observed in association with hepatocytic cell death (left panel). Vesicular SER and irregularly shaped mitochondria (M) with cristolysis, swelling and outer membrane rupture are depicted. (This figure appears in color on the web.)

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